Reconstructing phylogenetic relationship among Indian termite species inferred from COII gene sequences (Blattodea: Isoptera: Termitidae)

Mandakini Singla, Neha Goyal, Radhika Sharma, Nandita Singla, Anathbandhu Chaudhuri, RC Sobti and VL Sharma

Abstract
To analyze the phylogenetic relationship among nine species of Indian termites, 675 base pairs fragment of complete COII gene was utilized. Neighbor-joining (NJ) and Maximum Likelihood (ML) methods were used in Molecular Evolutionary Genetic Analysis (MEGA 5.2) software to define the molecular phylogeny. Both the trees revealed almost identical topology among the species and paraphyly of Microcerotermes and monophyly of the species belonging to subfamily Macrotermi tinae of family Termitidae were strongly supported by two reconstruction methods.

Keywords: Termitidae, COII, phylogeny

Introduction
Termites are group of social insects classified under Infraorder Isoptera, holding unresolved evolutionary origin for more than half a century [1-7] and include the most primitive insects of the terrestrial Neoptera, which also comprises cockroaches and mantids often embodied in a Superorder Dictyoptera and order Blattodea. Kambhampati et al. [8] carried out first molecular studies in Isoptera on a portion of the mitochondrial large ribosomal subunit gene (16SrRNA), in ten genera of five families to draw interfamilial relationships. Although different molecular markers were considered, the results obtained were similar [9]. The sequences of the mitochondrial genes have been widely used to estimate phylogenetic relationships at different taxonomic levels in insects [10-12]. Till now, the complete nucleotide sequences of the mitochondrial cytochrome oxidase II gene of 13 species of insects, representing 10 orders has been studied. The gene ranges from 673 to 690 bp in length encoding 226 to 229 amino acids. Several insertion or deletion events involving one or two codons can be observed. The 3' end of the gene is extremely variable in both the length and sequence [13]. The usefulness of COII region of the mitochondrial DNA (mt DNA) has been well demonstrated in studying the phylogenetic relationship of termites by some scientists [8, 14-24]. Mitochondrial genes are known to evolve more rapidly than nuclear genes and are therefore good markers to analyze relatively close relationships, such as species relationships within a genus [15, 23-28]. The protein coding COII gene of mitochondrial DNA has been used for many phylogenetic studies of termites from different geographical areas resulting in a large number of sequence data appearing in GenBank makes it is the most sequenced gene so far for the termites. We investigated the phylogenetic relationships among Indian termites of family Termitidae by using complete COII gene sequences.

2. Materials and Methods
2.1 Materials for molecular analysis
Termites for the present study were collected randomly from various regions of India as described in Table 1. Specimens were preserved in rectified alcohol and maintained in the Department of Zoology, Panjab University, Chandigarh (India). Soldier specimens were got identified from Zoological Survey of India, Kolkata and Forest Research Institute, Dehradun employing keys and descriptions of Chottani [29]. The details of collection site, date of collection, source and name of the collector were mentioned on each vial. The period of study, including collection, experimental designs, data analysis varied from 2010 to 2013.
2.2 DNA extraction, PCR and sequencing

Genomic DNA was extracted from worker termites by performing modified phenol: chloroform extraction method [30]. Polymerase Chain Reaction was conducted by applying protocol of Williams et al. [31] followed with some modifications. Approximately 780 bp fragment of COII gene flanking with tRNA’s was developed. Details of primers are as follows:

1. TL2-J-3037 (5-ATGGCA-GATTAGTGCAATGG-3) was designed by Liu and Beckenbach [13] and described by Simon et al. [32] and Miura et al. [14]. This is the 3037 primer, so named because of its location in the mt genome; this is partially overlapping with and complimentary to the COI primer 3041.

2. TK-N-3785 (5-GTTTAAGAGACCAGTACTTG-3) from Simon et al. [32], described by O’Grady et al. [33] and O’Grady [34]. This is the same as 3771 or 3791 based on its location in the mt genome.

These primers, originally published in Liu and Beckenbach [13], have been very useful for studying species and genus divergence in insects. Primers are anchored in transfer RNA genes flanking COII and are able to amplify the entire gene of approximately 673-690 in length. The amplified products were got sequenced directly from Chromous Biotech. Pvt. Ltd., Bangalore (India).

2.3 Phylogenetic analysis

The sequences were first edited manually for discarding the ambiguous and skipped bases and files were converted into FASTA format. The edited sequences were compared with related sequences from the nucleotide database of the National Centre for Biotechnology Information (NCBI) (http://www.ncbi.nlm.nih.gov), using Basic Local Alignment Search Tool (BLAST N) algorithm [35] to make sure that correct target sequences were amplified. Prior to tree building, all sequences were aligned automatically using the multiple alignment algorithm in Clustal omega [36] with the default settings. The evolutionary distances were computed using the Kimura 2-Parameter (K2P) method [37] and Evolutionary analyses were performed in MEGA 5.2 [38]. Phylogenetic analyses was performed employing the Neighbor-Joining (NJ) method based on the Kimura 2-Parameter distance with the uniform rate substitution among sequences and Maximum likelihood (ML) method by MEGA 5.2. Bootstrap analysis using 1000 pseudo replications [39] was included to test the reliability of inferred trees and all codon positions were included to verify the robustness of the internal nodes.

3. Results and Discussion

Average amplicon size resulting from DNA sequencing was 780 base pairs (bp). To facilitate genetic comparisons with existing GenBank DNA sequences, tRNA flanking with 5’end and 3’end of the amplicon was excluded, and the remaining 675 bp COII portion was used for further analyses.

3.1 Nucleotide analysis

The composition of the different nucleotides in the COII gene fragment of the nine species under study was noted and their percentages were calculated (Table 2).

### Table 1: Collection data of the Indian termite species (Family: Termitidae)

<table>
<thead>
<tr>
<th>S. No.</th>
<th>Identified Species</th>
<th>Collection Site</th>
<th>Family</th>
<th>Subfamily</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Microcerotermes beessonii (Snyder)</td>
<td>South Goa</td>
<td>Termitidae</td>
<td>Termitiniae</td>
<td>Cashew nut tree</td>
</tr>
<tr>
<td>2</td>
<td>Microtermes obesi (Holmgren)</td>
<td>Village Kothi Radha, Jagraon, Ludhiana Dist. (Punjab)</td>
<td>Termitidae</td>
<td>Macrotermiteinae</td>
<td>Damp wood</td>
</tr>
<tr>
<td>3</td>
<td>Microtermes unicolor (Snyder)</td>
<td>Malak Road, Jagraon (Punjab)</td>
<td>Termitidae</td>
<td>Macrotermiteinae</td>
<td>Log of wood</td>
</tr>
<tr>
<td>4</td>
<td>Microtermes mycophagus (Desneux)</td>
<td>Badelar, Hammerpur Dist. (H.P.)</td>
<td>Termitidae</td>
<td>Macrotermiteinae</td>
<td>Tree trunk</td>
</tr>
<tr>
<td>5</td>
<td>Odontotermes horni (Wasmann)</td>
<td>Sec.11, Chandigarh</td>
<td>Termitidae</td>
<td>Macrotermiteinae</td>
<td>Damp wood, tree</td>
</tr>
<tr>
<td>6</td>
<td>Odontotermes obesus (Rambur)</td>
<td>Mandi (Hischal Pradesh)</td>
<td>Termitidae</td>
<td>Macrotermiteinae</td>
<td>Mound</td>
</tr>
<tr>
<td>7</td>
<td>Odontotermes gurdsapurensis</td>
<td>Pipli, Haryana</td>
<td>Termitidae</td>
<td>Macrotermiteinae</td>
<td>Wood</td>
</tr>
<tr>
<td>8</td>
<td>Odontotermes brunus (Hagen)</td>
<td>Sec. 10, Chandigarh</td>
<td>Termitidae</td>
<td>Macrotermiteinae</td>
<td>Tree</td>
</tr>
</tbody>
</table>

The COII gene fragment showed high percentage of A+T that varied from 59.72 to 60.86. Nucleotide variation showed an adenine thymine bias in their composition that is consistent with data on other insect COII mitochondrial gene studies [40-42]. Figure 1 clearly indicates the same observations.

### Table 2: Percentage of nucleotides in COII gene fragment in termites species under study

<table>
<thead>
<tr>
<th>S. No.</th>
<th>Identified Species</th>
<th>A+T (%)</th>
<th>G+C (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Microteremis beessonii</td>
<td>60.75</td>
<td>39.25</td>
</tr>
<tr>
<td>2</td>
<td>Microtermes obesi</td>
<td>60.17</td>
<td>39.83</td>
</tr>
<tr>
<td>3</td>
<td>Microtermes unicolor</td>
<td>60.02</td>
<td>39.98</td>
</tr>
<tr>
<td>4</td>
<td>Microtermes mycophagus</td>
<td>60.72</td>
<td>39.28</td>
</tr>
<tr>
<td>5</td>
<td>Odontotermes horni</td>
<td>59.65</td>
<td>40.35</td>
</tr>
<tr>
<td>6</td>
<td>Odontotermes obesus</td>
<td>59.72</td>
<td>40.28</td>
</tr>
<tr>
<td>7</td>
<td>Odontotermes gurdsapurensis</td>
<td>60.72</td>
<td>39.28</td>
</tr>
<tr>
<td>8</td>
<td>Odontotermes brunus</td>
<td>60.86</td>
<td>39.14</td>
</tr>
<tr>
<td>9</td>
<td>Odontotermes bhagwattai</td>
<td>59.87</td>
<td>40.13</td>
</tr>
</tbody>
</table>

3.1.1 Species of termites under study

As proven by other researchers, a phylogenetic analysis of Australian dry wood termites was done by Thompson et al. [43] utilizing 624 bp long fragment of COII gene which showed an adenine thymine bias in the nucleotide composition (A+T=64.2%). Based on the DNA sequence of a portion of the mitochondrial COII gene of Reticulitermes including 21 species and subspecies from three continents, the average base
frequencies were found to be A+T=63% and G+C=37%. [22],

Four species belonging to the genus Odontotermes (O. obesus, O. horni, O. bhagwatii and Odontotermes sp.) and one of Microtermes (M. obesi), a high A + T content was observed [44]. A + T content was found to be 59.55% and G + C = 40.45% for Microtermes obesi.

3.2 Pairwise genetic distance

The sequences of the complete fragment of COII gene from the termites species under study were used to calculate Pairwise genetic distance values (Kimura 2 parameter) using MEGA 5.2 (Table 3).

### Table 3: Pairwise genetic distances (Kimura 2-parameter) in species under study

<table>
<thead>
<tr>
<th>S. No.</th>
<th>Identified Species</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
<th>7</th>
<th>8</th>
<th>9</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Odontotermes bhagwati</td>
<td>0.063</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>Odontotermes brunneus</td>
<td>0.068</td>
<td>0.181</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>Microtermes unicolor</td>
<td>0.186</td>
<td>0.191</td>
<td>0.060</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>Odontotermes gudapsensis</td>
<td>0.072</td>
<td>0.060</td>
<td>0.196</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>Odontotermes horni</td>
<td>0.057</td>
<td>0.025</td>
<td>0.174</td>
<td>0.044</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>Odontotermes obesus</td>
<td>0.061</td>
<td>0.056</td>
<td>0.187</td>
<td>0.058</td>
<td>0.043</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>Microtermes mycophagus</td>
<td>0.127</td>
<td>0.134</td>
<td>0.054</td>
<td>0.139</td>
<td>0.117</td>
<td>0.128</td>
<td></td>
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<td></td>
</tr>
<tr>
<td>8</td>
<td>Microtermes obesi</td>
<td>0.157</td>
<td>0.165</td>
<td>0.030</td>
<td>0.167</td>
<td>0.145</td>
<td>0.161</td>
<td>0.028</td>
<td></td>
<td></td>
</tr>
<tr>
<td>9</td>
<td>Microcerotermes beesoni</td>
<td>0.194</td>
<td>0.212</td>
<td>0.229</td>
<td>0.200</td>
<td>0.199</td>
<td>0.199</td>
<td>0.181</td>
<td>0.182</td>
<td>0.192</td>
</tr>
</tbody>
</table>

The K2P distance in congeneric species of genus Odontotermes varies from 0.025 to 0.072. It was found to be lowest (0.025) between O. brunneus and O. horni and highest (0.072) between O. bhagwati and O. gudapsensis. The interspecies K2P distance of genus Microtermes was highest (0.054) between M. unicolor and M. mycophagus and lowest between (0.026) between M. mycophagus and M. obesi. Microcerotermes beesoni gave highest value with the other species under study. K2P distance of M. beesoni was highest (0.229) with M. unicolor and lowest (0.181) with "M. mycophagus.

3.3 Phylogenetic inferences

Phylogenies are helpful for organizing knowledge of biological diversity, describing structuring classifications and providing insight into the events that occurred during evolution. The sequences of complete COII gene fragment were compared with the sequences available in the database (Table 4) retrieved from National Centre for Biotechnology Information (NCBI). The species retrieved from GenBank were given in both the trees with their accession numbers in parentheses. By using MEGA 5.2 software, phylogenetic trees were drawn by NJ (Fig. 2) and ML (Fig. 3) methods.

### Table 4: Species data summary included in the phylogenetic analysis; References are given for sequences obtained from GenBank

<table>
<thead>
<tr>
<th>S. No.</th>
<th>Taxon</th>
<th>Family</th>
<th>Subfamily</th>
<th>Locality</th>
<th>Accession number</th>
<th>Author</th>
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<td>1</td>
<td>Odontotermes bhagwati</td>
<td>Termitidae</td>
<td>Macrotermiteinae</td>
<td>India</td>
<td>EU242525</td>
<td>Sobti et al. [44]</td>
</tr>
<tr>
<td>2</td>
<td>Odontotermes brunneus</td>
<td>Termitidae</td>
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<td>India</td>
<td>EU242523</td>
<td>Sobti et al. [44]</td>
</tr>
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<td>3</td>
<td>Hypotermes mahanensis</td>
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<td>Thailand</td>
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</tr>
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</tr>
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</tr>
<tr>
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<td>Macrotermiteinae</td>
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<td>EU242522</td>
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</tr>
<tr>
<td>22</td>
<td>Microtermes obesi</td>
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</tr>
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</tbody>
</table>
The topology of both the trees (NJ, ML) was nearly identical, differing basically in supporting values of nodes. Our phylogenetic analyses defined four robust clades: one (cluster I) included 21 sequences representing all members of genus Odontotermes and two species of Hypotermes. The second (Cluster II) comprised of Pseudocanthotermes and third (Cluster II) comprised Ancistrotermes. Fourth (cluster IV) included 11 sequences representing all members of genus Microtermes. All the four clusters comprised of the species belong to subfamily Macrotermitinae of family Termitidae. Within first cluster, two strongly supported subclades were resolved as indicated by high bootstrap value. One subclade was mostly composed of the oriental species of genus Odontotermes and Hypotermes while the second comprised the Palearctic genera Odontotermes with one Oriental species. In cluster II and III, species respectively belonging to the genus Pseudocanthotermes and Ancistrotermes formed their own clusters. In cluster IV, two subclades were resolved belonging to genus Microtermes: one subclade comprising of Oriental species and other comprising of all Afrotropical species with one species of oriental origin (Microtermes pakistanicus).

A sharp cluster representing subfamily Termitinae of genus Microcerotermes was observed at the bottom of both the trees indicating Termitinae to be paraphyletic. All the congeneric species supported a good bootstrap value indicating genetic relationship between them. The conspecific individuals of genus O. horni, O obesus, O. bhagwaitii and M. obesi were supported by more than 85% bootstrap values. All the congeneric species and conspecific individuals clustered together indicating higher bootstrap values.

A comprehensive phylogenetic analysis of the family Rhinotermitidae as a whole has been carried out by Austin et al. [51] by using the DNA sequences of a portion of mitochondrial COII gene revealing sufficient variation in many species from North America, Europe and Asia [45]. The amount of genetic differentiation among several Reticulitermes lucifugus populations was also determined [52] and this study has supported the view that Mastotermitidae is the basal lineage among extant termites and the family Rhinotermitidae is polyphyletic, given the familial status of Serritermitidae. Moreover, distinct groups were established within this family by studies of other scientists [46, 53-58].

Also morphological identifications of Malaysian termites in the family Termitidae (Isoptera) were verified by Lee et al. [59] by phylogenetic analyses of COII gene sequence. The extent of genetic relatedness/variations within and between various populations of the different species of Indian termites belonging to the family Termitidae was observed by using 12SrRNA, 16S rRNA, COI, COII, NDI genes resulting in dissonant results indicates the usefulness of complete genome analysis of these species [44, 60-66].

Also discussed by Lo et al. [67], some Australian termites to be more closely related to Asian termites than to other species native to Australia, again suggesting the possibility of over water movement for a short distance. Coptotermes formosanus population of Taiwan, Japan and China and C. gestroi populations of Taiwan showed near resemblance with those of Philippines and Hawaii as observed by Li et al. [68]. Phylogenetic tree drawn on the basis of combined nucleotide matrices of 12S, 16S and COII gene among 11 subterranean termite species of Coptotermes from East Asia and Australia revealed two major clades with six subclades [69] showing relatedness among these species.

Thus, it can be inferred that these phylogenetic trees have been
an essential tool in the study of evolution since the time of Charles Darwin. It clearly depicts the utility of such genetic tool in establishing the overall picture of relationship and taxonomic positioning of the lesser known species. As expected, the present analysis revealed phylogenetic relatedness either on the basis of genera, species or geographical location and it seems that all these species had common origin but later got diversified with the change in geographical location. Today, similarities in lower termite species across continents such as Australia, South America, and Africa indicated that they retain a genetic link from the time they were all joined as Gondwana.

4. Conclusion
The analysis will be valuable in studying molecular taxonomy particularly for those species that are difficult to identify using morphologic characters. Phylogeny can help indicating the identity of the unidentified species but can further be confirmed by morphological analysis using DNA barcoding. Our construction of phylogeny emphasizes the strikingly different species of termites showing genetic relationship with each other belonging to different geographical regions. This profound evolutionary relationship of Indian termites can be further exploited for comparison with other gene sequences.

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